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Stealth® liposomal tumor necrosis factor-α in solid tumor treatment

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Abstract

Tumor Necrosis Factor-α is a cytokine with proven antitumor effects. Due to severe side effects however, systemic treatment with TNF-α is not possible. Here we discuss the possible usefulness of Stealth® liposome-technology for successful application of TNF-α for the treatment of solid tumors. Tumor Necrosis Factor-α has been applied successfully in combination with melphalan and interferon-γ in isolated limb perfusion in patients with high grade malignant melanoma and irresectable soft tissue sarcoma. For tumors located in organs, isolated perfusion with TNF-α seems attractive. However, isolated organ perfusion is technically demanding and might cause severe morbidity. Moreover, treatment of metastatic disease requires systemic application. One way of doing this is the application of TNF-α in liposomes, injected systemically. The encapsulation of TNF-α in conventional, large multilamellar liposomes leads to less toxicity, but antitumor treatment was disappointing, due to rapid recognition of the liposomes by cells of the mononuclear phagocyte system. Probably, encapsulation of TNF-α in Stealth® liposomes is more promising in treatment of solid organ tumors and disseminated disease. Possible use of these liposomes is shown on the basis of results obtained in two rat tumor models with Stealth® liposome encapsulated Tumor Necrosis Factor-α in combination with Stealth® liposome encapsulated doxorubicin (DOXIL®).

Keywords: Stealth®; Liposomes; TNF-α; Cytotoxic agents; Solid tumor; Rats

1. Introduction

Tumor Necrosis Factor-α is a pluripotent cytokine with immunomodulatory, inflammatory and antitumor effects (Carswell et al., 1975). It is mainly produced by activated monocytes and macrophages. TNF-α is a trimer, which binds in

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vivo to two receptors, a 55 and a 75 kD receptor, of which the former is held responsible for the direct cytotoxic effect of TNF-α. The indirect antitumor effects of TNF-α however, are more prominent. The vascular endothelium of the tumor acts as an effector organ, leading to von Willebrand factor release (Renard et al., 1995). TNF-α induced upregulation of adhesion molecules, increased microvasculature permeability and conversion of tumor endothelial cells into a procoagulant state has been described (Shimomura et al., 1988; Watanabe et al., 1988). By acting on permeability and integrity of the tumor neovasculature, accompanied with platelet aggregation, TNF-α induces edema and hemorrhagic necrosis of the tumor (Nooijen et al., 1996).

Tumor Necrosis Factor-α has impressive antitumor activity in clinical and preclinical setting. In patients with advanced melanoma isolated limb perfusion (ILP) with high dose TNF-α in combination with melphalan an 80% complete remission (CR) and a 15% partial remission (PR) rate has been observed (Liénard et al., 1992, 1994). In patients with large soft tissue sarcomas, a 29% CR and a 53% PR was reached, resulting in an 82% limb salvage rate (Eggermont et al., 1993, 1996b). Animal studies revealed synergistic antitumor effects of TNF-α and melphalan in isolated limb perfusion (Manusama et al., 1996a,b). Thus, combining TNF-α and cytotoxic agents is mandatory for tumor regression. Moreover, antitumor activity in vivo was only observed with high dosages of TNF-α ($50 \times$ maximum tolerated dose, MTD). This high dose TNF-α could only be reached during ILP. Systemic treatment with TNF-α is therefore, not only hampered by the fast clearance of the drug and poor localization in tumor tissue, but also by severe toxic side effects (Tracey et al., 1986; Asher et al., 1987). These drawbacks make effective systemic treatment with free TNF-α impossible.

For tumors located in organs, a similar approach as isolated limb perfusion is attractive. However, isolated perfusion of organs is technically demanding and causes considerable morbidity (Pogrebniak et al., 1994; van der Veen et al., 1994, Weksler et al., 1994; Pass et al., 1996; Borel Rinkes et al., 1997). Moreover, treatment of metastatic disease requires systemic application.

In finding strategies to deliver TNF-α at the tumor site, liposomes are suggested as controlled release vehicles. Various formulations of liposomes have been described. In this review, liposomal encapsulation of Tumor Necrosis Factor-α will be described. The possible advantages of liposomal delivery will be covered. Furthermore, the applications of liposomal TNF-α in combination with Stealth® liposomal doxorubicin in solid rat tumors will be described.

2. Tumor necrosis factor-α, preclinical and clinical studies

Due to the severe toxicity, the clinical application of TNF-α proved to be disappointing. Phase I–II studies revealed extremely low remission rates with the maximal tolerated dose in humans of $\pm 350\ \mu g/m^2$ i.v. (Feinberg et al., 1988; Spriggs et al., 1988). A 10–50-fold higher dose would be necessary to achieve antitumor effects (Asher et al., 1987). TNF-α was therefore restricted to intratumoral or intracavitary usage, thus allowing for high local concentrations.

High locoregional concentrations of TNF-α were also reached with isolated limb perfusion. A major increase in concentration of TNF-α of 15–20 times the concentrations reached in systemic application was observed. One must keep in mind that these concentrations are only reached for a relatively short period of time, e.g. the time during which the perfusion is performed (30–90 min).

The results of isolated limb perfusion with TNF-α and melphalan for high grade melanoma and irresectable sarcoma have recently been published (Liénard et al., 1992; Eggermont et al., 1993, 1996a,b). A high complete remission rate was reported for patients with in transit metastases from malignant melanoma with the aforementioned treatment in combination with interferon-γ. The same regimen for patients with a nonresectable extremity soft tissue sarcoma yielded a limb salvage rate of 82%.

To examine whether synergism between TNF-α and melphalan played a role in the observed anti-tumor effect, pre-clinical studies were started at our laboratory. In vivo, a synergistic effect was

proven on a soft tissue sarcoma as well as an osteosarcoma model in the rat with 40 μg melphalan and 50 μg TNF-α (Manusama et al., 1996a,b). It was suggested that indirect effects of TNF-α played a crucial role. High dose TNF-α was shown to induce destruction of endothelial lining, coagulation and thrombus formation in tumor associated vasculature with stasis of erythrocytes and platelet aggregation (Renard et al., 1995; Nooijen et al., 1996).

2.1. Liposomal encapsulation of TNF-α, conventional liposomes

To address the main adverse effect of Tumor Necrosis Factor-α, its severe toxicity, liposomal encapsulation was proposed. Liposomal preparations of TNF-α have been described by several authors (Debs et al., 1989; Düzgünes et al., 1989; Debs et al., 1990; Nii et al., 1991; Utsumi et al., 1991; Weber et al., 1994; Lodato et al., 1995; Kedar et al., 1997). The liposomes used in these studies were of the conventional type: oligo- or multilamellar vesicles with a diameter of >200 nm. These liposomes however, are rapidly recognized by cells of the mononuclear phagocyte system (MPS), and are therefore, characterized by a short circulation time and hence a disappointing localization in tumors. Size, lipid composition and route of injection were varied to achieve better results, but the conventional liposomes were deemed to be used only in malignancies of the reticuloendothelial system, especially in organs such as the liver, spleen and lymph nodes.

Encapsulated TNF-α retained immunomodulatory and antitumor activity in vivo, at the same time reducing toxicity compared to free TNF-α in rats (Debs et al., 1990). For alteration of the biological activity of TNF-α, stable association on or within the liposomal membrane appears mandatory. This was further proven by the fact that liposomal TNF-α retains bioactivity in vitro in the presence of anti-TNF-α antibodies (Debs et al., 1989). Sensitive target cells, like the human melanoma A375 and the murine fibroblasts L929, were effectively lysed by TNF-α entrapped in liposomes (Nii et al., 1991). Others found that liposomes loaded with TNF-α had a growth inhibitory effect on human glioma cells, a cell line which is resistant to free TNF-α. However, the TNF-α-liposomes did not augment the inhibiting effect of empty liposomes. It was suggested that the liposomal membrane itself slowed the growth of the cells and TNF-α bound to the liposomes was not active (Weber et al., 1994).

From these and other studies it is believed that TNF-α has to bind to plasma membrane expressed receptors to be active, whereas intracellular delivered TNF-α is not. Therefore, we hypothesize that optimal antitumor activity with liposomal TNF-α will be reached when TNF-α is released after extravasation in tumor tissue, but before degradation of the liposomes occurs. Others postulated that TNF-α which is entrapped in the aqueous compartment of liposomes, is incapable of reacting with the cell surface receptor. Thus, the phospholipid membrane is blocking the direct cytotoxic mechanism of TNF-α (Kedar and Barenholz, 1997).

It was soon recognized that encapsulation of TNF-α in liposomes was hampered by the low hydrophobicity of the cytokine. The preparation of a more lipophilic variety of the native TNF-α was therefore attempted. Incorporation of TNF-α coupled to long chain fatty acids proved to be successful in small unilamellar vesicles (Utsumi et al., 1991; Klostergaard et al., 1992). The biological activity however, was negatively influenced by modification of the amino residues in TNF-α, thought to be essential for its cytolytic effect. Nevertheless, the binding efficiency was markedly improved ($>50\%$), and a possible usage was foreseen in treating tumors. Encapsulation of TNF-α in liposomes was also enhanced by varying the calcium concentration in the membrane (Saito et al., 1995). Supposedly, negatively influencing the stability of the phospholipid membrane played a role in the process. By combining interleukin-1α and TNF-α in liposomes metastases of a melanoma cell line were reduced, a mechanism not well understood.

Conjugation of tissue specific antibodies to conventional liposomes has been tried, but targeting in vivo has been unsuccessful due to rapid MPS uptake (Allen and Moase, 1996), even though in vitro cells normally resistant to TNF-α became

sensitive (Morishige et al., 1993). Antibody-mediated targeting of liposomes with MPS avoiding characteristics (which are discussed below) might provide tools for the production of immunoliposomes which still exhibit long circulating times, but also have the ability to recognize the tissue of interest (Allen, 1996). However, incorporation of antibodies enhance recognition by the MPS, and moreover bind to tumor cells just outside the vascular wall. Further penetration of the liposomes might be thus prevented, with heterogeneous distribution and diminished antitumor activity, especially in large solid tumors.

In summary, TNF-α encapsulated in conventional liposomes is rapidly recognized by cells of the MPS, and therefore less useful in treating non RES tumors. We therefore believe that, for the treatment of large solid tumors, with a highly developed vasculature, the use of MPS avoiding liposomes is preferred over conventional liposomes, with or without coupled antibodies.

2.2. Liposomal encapsulation of TNF-α, Stealth® liposomes

The liposomal TNF-α formulations described above are multilamellar larger liposomes. By the early nineties a new generation of liposomes became known as long circulating (sterically stabilized or Stealth®) liposomes (Papahadjopoulos et al., 1991; Woodle and Lasic, 1992). These liposomes were characterized by longer blood circulation times, due to a lesser uptake by the MPS (Gabizon and Papahadjopoulos, 1992). By incorporation of a polyethylene glycol (PEG), coupled to a phospholipid (for example phosphatidylethanolamine, PE) a watermantle was created around the liposome (Klibanov et al., 1990; Woodle and Lasic, 1992). Thus, opsonization by plasma proteins was less and recognition by the immune system was accordingly avoided. Stealth® liposomes have a diameter < 100 nm, which allows extravasation through the endothelial gaps in the microvasculature of the tumor (Huang et al., 1992; Jain, 1994). Increased permeability of microvasculature contributes to the accumulation of these liposomes in the tumor (Wu et al., 1993). After promising phase I–II studies (Gabizon et al., 1994), the production of doxorubicin in Stealth® liposomes was started (DOXIL®, SEQUUS Pharmaceuticals, Menlo Park, CA), which is now registered for the treatment of refractory Kaposi sarcoma in the USA.

Our laboratory began to examine whether it was possible to encapsulate Tumor Necrosis Factor-α in Stealth® liposomes. We demonstrated that TNF-α could indeed be incorporated in these long circulating liposomes with good encapsulation efficiency (ten Hagen et al., 1996). These liposomes, called TNF-SL, were therefore used in biodistribution experiments. We showed that the blood circulation of TNF-SL was comparable to the circulation times mentioned in the literature (Gabizon et al., 1994; Harashima et al., 1996; van der Veen et al., 1996). Empty liposomes demonstrated typical Stealth® characteristics with a half life time in blood of ≈ 22 h, and low uptake in MPS rich tissues. TNF-α encapsulated liposomes had a slightly shorter residence half-life (ten Hagen et al., 1997), probably caused by in vivo release of TNF-α from the liposomes. This phenomena is also known for IL-2 encapsulated in sterically stabilized liposomes (Kedar et al., 1994). A total of 72 h after injection of liposomes, 15% of the injected dose could still be detected in the circulation. The liposomes used showed localization in a soft tissue sarcoma in the leg, a tumor model also used in isolated limb perfusion studies, of almost 9% of the injected dose, 24 h after injection. Comparable to the results shown with TNF-α entrapped in conventional liposomes, toxicity with TNF-SL was markedly reduced. No loss in weight was recorded.

Subsequently, in the soft tissue sarcoma we tested the antitumor efficacy of different combinations of TNF-SL and doxorubicin encapsulated in Stealth® liposomes (DOXIL®, kindly provided by Dr Working, SEQUUS Pharmaceuticals, Menlo Park, CA, USA). Results are shown in Table 1. With the combination of TNF-SL and DOXIL®, injected five times over a 20 day period, we observed a good tumor response (van der Veen et al., 1996). All the tumors showed at least a stable disease for a prolonged period of time. In a number of tumors, the tumor, but also the overlying skin, became necrotic. In histopathologic

studies, this region of necrosis was marked, albeit not a 100%. A viable rim was seen around the necrotic region (ten Hagen et al., 1997).

Efficacy of TNF-SL was also studies in a liver tumor model. The BN 175 soft tissue sarcoma, placed under the liver capsule, showed good growth curves when untreated. The same regimen used for the treatment of the tumor in the limb, was used to treat the liver tumor. Results are shown in Table 2. Although a response to treatment with DOXIL® in combination with empty liposomes is seen, a better growth inhibiting effect is seen when this tumor is treated with DOXIL® in combination with TNF-SL.

3. Summary and conclusions

Isolated limb perfusion with Tumor Necrosis Factor-α in combination with melphalan and interferon-γ showed high remission rates in patients with advanced melanoma and irresectable sarcoma (Liénard et al., 1992; Eggermont et al., 1993, 1996a,b). Synergism between TNF-α and melphalan in ILP was seen in two different sarcoma tumor models used in our laboratory (Manusama et al., 1996a,b). Liposomes were used as a drug delivery system, but for the treatment of malignancy conventional liposomes were not very promising. Although liposomes are biocompatible, biodegradable and show low inherent toxicity, the physical characteristics of the earlier generations of liposomes were such that rapid recognition by cells of the mononuclear phagocyte system took place, with low residence half-times as a consequence. Sterically stabilized or Stealth® liposomes have been shown to stay in the circulation for relatively long periods of time. Depending on the physicochemistry of the liposomes and on the animal model used, half life times of 20–40 h are described (Gabizon et al., 1994; Harashima et al., 1996; van der Veen et al., 1996). Delivery of various kinds of cytokines (IL-1, IL-2, IL-6, TNF-α, IFN-α, IFN-γ and GM-CSF) in liposomes have been reported (van der Veen et al., 1996; ten Hagen et al., 1997; Kedar and Barenholz, 1997), and it is expected that the therapeutic index of tumor treatment with these liposomes is widened.

Encapsulation of TNF-α therefore has advantages, depending on the type of liposome used. Conventional liposomal TNF-α has been shown to lower the toxicity of TNF-α. Cytotoxicity of TNF-α in vitro is unaffected by entrapment in liposomes, however conflicting results have been reported. We believe that TNF-α has to be re-

Table 1
Tumor response in rats[a]

Treatment	Response rate[b]	Response type[c]
TNF free	0	PD
TNF-SL	0	PD
DOXIL	0	PD
TNF-SL + DOXIL	100%	SD

Small tumor fragments of the BN175 soft tissue sarcoma were implanted subcutaneously in the right hind leg of rats. Treatment (5 injections, q 4 days) was started at a tumor diameter of 10 mm. Tumor diameter changes $<25\%$ were classified as SD.

[a] To systemic treatment with Stealth® liposome-encapsulated Tumor Necrosis Factor-α (TNF-SL) and Stealth® liposome-encapsulated doxorubicin (DOXIL®).

[b] Percentage of rats responding to treatment.

[c] PD, progressive disease; SD, stable disease (>14 days).

Table 2
Tumor response of BN 175 sarcoma[a]

Treatment	Tumor diameter (mm) day 7	Tumor diameter (mm) day 25
Sham	6.5	nm[c] (13)[d]
TNF-SL + Pl[b]	7	nm (14)
DOXIL + PL	8	nm (17)
DOXIL + TNF-SL	6	19

Tumor was implanted in the main liver lobe of rats and tumor diameter recorded with an interval of 5–7 days. Treatment (5 injections, q 4 day) was started at a tumor diameter of 6–8 mm.

[a] Placed under the liver capsule in rats to systemic treatment with Stealth® liposome-encapsulated Tumor Necrosis Factor-α (TNF-SL) and Stealth® liposome-encapsulated doxorubicin (DOXIL®).

[b] PL, placebo liposomes.

[c] nm, Not measurable. Animal sacrificed at tumor diameter >20 mm.

[d] Average day after implantation at which rats were sacrificed.

leased from the liposomes, followed by binding to the extracellular receptor to be active. Also, combination of TNF-α with conventional cytotoxic agents for treatment of solid tumors seems necessary. Although systemic toxicity normally seen with the free agent is abolished by liposomal encapsulation, other types of toxicity might be seen in return (i.e. reduced cardiac toxicity with liposomal encapsulation of doxorubicin, but increased toxicity to skin and tissue macrophages at high dosages (Gordon et al., 1995)). The liposome type of choice, as well as the treatment protocol, therefore depends on the cytotoxic agent used and the tumor type to be treated. In case of agents with cytotoxicity at the DNA level, cellular uptake is mandatory, whereas TNF-α, which is most likely active through the extracellular receptor, is preferably released in the vicinity of the cells.

Here we discuss the applicability of liposomal TNF-α in cancer research. Its major advantage is decreased toxicity, with increased bioavailability. Thus far, in our studies with Stealth® liposome encapsulated TNF-α a 10–20-fold increase in area under the curve (AUC) could be achieved. However, unknown is whether the TNF-α is released or still associated with the liposomes determining its bioavailability. Conventional liposomes are not suitable for systemic disease, nor for the treatment of large solid tumors. To replace the isolated perfusion and to treat metastases, therefore, incorporation of TNF-α in long circulating liposomes is more promising, especially when combined with cytotoxic agents encapsulated within liposomes like doxorubicin.

We anticipate that these liposomes will be soon incorporated in anti-cancer biotherapy, not to replace the isolated perfusion techniques, but as a useful adjunct to systemic disease.

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